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Carbohydrate and organic acid composition of effective and ineffective root nodules of *Phaseolus vulgaris*

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Dicarboxylic acid transport mutants of *Rhizobium* species are usually deficient in their ability to fix atmospheric dinitrogen. We report here a study comparing the physiology of root nodules on *Phaseolus vulgaris* L. cv. Goldie induced by an effective strain of *Rhizobium leguminosarum* biovar *phaseoli* and a C_4-dicarboxylic acid utilization mutant. The mutant, while able to form nodules, was ineffective in N_2 fixation. Carbohydrates and organic acids of roots and nodules formed by the 2 strains were monitored at 3-day intervals from 13 to 34 days after inoculation. Both carbohydrates and organic acids accumulated in ineffective nodules in comparison with the effective nodules. The concentration of malic acid was tenfold higher in ineffective nodules than in effective nodules. Other organic acids, i.e., lactate, malonate, ascorbate and gluconate, were also detected. Lactate and ascorbate were the only other organic acids accumulating in ineffective nodules. The most prevalent carbohydrates found in both types of nodules were sucrose, glucose and fructose. Myo-inositol was the only cyclitol detected in both types of nodules. Carbohydrates and organic acids were present in lower concentration in roots than in nodules, except for lactate. These compounds were not consistently detected in higher concentration in roots from plants inoculated with the mutant strain, as was the case in nodules.

Key words – C_4-dicarboxylic acid mutant, French bean, *Rhizobium,* symbiosis, legume.

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Introduction

Symbiotic dinitrogen fixation by the *Rhizobium*-legume and *Bradyrhizobium*-legume symbioses involves complex metabolic interactions between the cytosol of root nodule cells and N_2-fixing bacteroids. It is well established that photosynthesis supports N_2 fixation (Pate 1977). It is not clear which compounds, supplied by plant cells to bacteroids, are necessary to satisfy the energy requirements for symbiotic N_2 fixation. In efforts to understand the metabolic interactions between bacteroids and plant cells, several studies have utilized mutant rhizobial strains. Mutants of *R. leguminosarum* biovar *viceae* (Finan et al. 1983, Arwas et al. 1985), *R. leguminosarum* biovar *trifolii* (Ronson et al. 1981) and *R. meliloti* (Bolton et al. 1986, Lafrenière et al. 1987) defective in dicarboxylic acid transport all induced ineffective nodules. On the other hand, mutants of *R. leguminosarum* biovar *viceae* (Glenn et al. 1984, Arwas et al. 1986) and *R. leguminosarum* biovar *trifolii* (Ronson and Primrose 1979) deficient in carbohydrate utilization retained their ability for symbiotic N_2 fixation. However, one exception was observed with a fructokinase mutant of *R. meliloti* inducing ineffective nodules (Duncan 1981). This indicates that the C_4-dicarboxylic acid intermediates are the most probable source of energy supplied by plant cells to bacteroids. In fact, many studies show that isolated bacteroids can take up (DeVries et al. 1982, Reibach and Streeter 1984, Saroso et al. 1984, San Francisco and Jacobson 1985) and oxidize

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(Salminen and Streeter 1987) C_4-dicarboxylic acids. None of these studies have addressed the nature and concentration of particular metabolites in effective and ineffective nodules formed by a C_4-dicarboxylic acid mutant.

The purpose of the present study was to compare the carbohydrate and organic acid contents of French bean (*Phaseolus vulgaris* L.) nodules formed by the effective *R. leguminosarum* biovar *phaseoli* strain P_{121} and its ineffective C_4-dicarboxylic acid mutant $P_{121}S_{21}$.

Abbreviations – ARA, acetylene reduction activity; DAI, days after inoculation.

Materials and methods

Bacterial strains

Two strains of *Rhizobium leguminosarum* biovar *phaseoli*, P_{121} and $P_{121}S_{21}$, were used in this study. The wild type strain, P_{121}, was isolated from Quebec soils and was classified as a very effective strain on French bean (*Phaseolus vulgaris* L. cv. Goldie) (Lalande et al. 1986). Following N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis (Lafrenière et al. 1987) a succinate, fumarate, malate utilization negative mutant, $P_{121}S_{21}$, was isolated. Bacteria were preserved at −80°C in yeast extract-mannitol broth (Vincent 1970) containing 10% glycerol.

Plant material and growth conditions

Seeds of French bean (*Phaseolus vulgaris* L. cv. Goldie) were surface sterilized in 6% sodium hypochlorite for 10 min, followed by 6 rinses in sterile water. Seeds were germinated on sterile cotton for 3–4 days before planting in plastic pots containing sterilized vermiculite. After emergence, seedlings were carefully removed from the pots and inoculated by soaking the roots for 1 min in a fresh liquid culture of strain P_{121} or strain $P_{121}S_{21}$ (10^8–10^9 cells ml^{-1}). Inoculated seedlings were then immediately transferred to a hydroponic growth system, which consisted of a 1 l glass jar containing ca 500 ml of nitrogen-free nutrient solution (Hoagland and Arnon 1938) with the following additions: 1 m*M* K_2HPO_4; 1 m*M* $CaCl_2$ and 0.017 μ*M* $CoCl_2.6H_2O$. The nutrient solution was aerated with a gas-dispersion tube. The jar was sealed with a cork cap bearing holes for aeration (i.e., air tubing) and plant growth (2 plants per jar). The whole system was autoclaved prior to plant insertion and remained free of contamination throughout the duration of the experiment. Plants were grown in a glass house at 23 ± 2°C and natural daylight was supplemented with a 16 h photoperiod under cool white fluorescent lamps (General Electric, Montreal, Que., Canada), 600 μmol m^{-2} s^{-1} quantum flux density. Plants were harvested 13, 16, 19, 22, 25, 28, 31 and 34 days after inoculation (DAI). Acetylene reduction activity (ARA) was determined. Plants were then separated into nodules, roots, stems and leaves and weighed. Plant parts were immediately frozen and then lyophilized. The experiment was a randomized complete block design with 3 replications. Each experimental unit consisted of 1 jar (2 plants).

Acetylene reduction activity

ARA was determined on excised roots of 2 plants in a 150 ml glass jar with a 10% (v/v) acetylene: air atmosphere. Gas samples were taken after a 30 min incubation and ethylene concentration was measured with a Perkin Elmer (Montreal, Que., Canada) Sigma 3B dual FID gas chromatograph, equipped with a 1 m stainless steel column packed with Porapak R (80–100 mesh). The column temperature was maintained at 45°C with a N_2 flow rate of 13 ml min^{-1}. The injector was maintained at 55°C and the detector at 125°C.

Extraction and analysis of carbohydrates and organic acids

Lyophilized samples of nodules and roots were ground and stored at −20°C under nitrogen. Extractions were carried out by adding 3 ml of 80% (v/v) ethanol to 10 and 20 mg samples of nodule and root tissue, respectively. Extractions were performed overnight (16h) at 30°C with agitation (200 rpm). Aliquots (0.5 ml) of the crude ethanolic extracts were evaporated to dryness under a flow of N_2. Formation of the trimethylsilyl-derivatives of carbohydrates and organic acids was performed by dissolving the dried extract in 100 μl of a 4:2:1 mixture (v/v/v) of pyridine, hexamethyldisilazane and trimethylchlorosilane (Pierce Chemical Co., Rockford, IL, USA), and agitating overnight (16 h) at 30°C. Carbohydrates and organic acids were analyzed by gas-liquid chromatography with a Perkin Elmer gas chromatograph, model Sigma 3 B, equipped with a dual flame ionization detector. Aliquots (2 μl) from the derivatized extracts were injected into a 2 m × 2 mm i.d. glass column, packed with 3% SE-52 on Chromosorb W-HP (100–120 mesh) with a N_2 flow rate of 18 ml min^{-1}. The injector was maintained at 240°C and the detector at 300°C. After an initial hold of 5 min at 90°C, the column was temperature-programmed at 5°C min^{-1} up to 270°C, which was held for 5 min. Peak integration was performed by a Hewlett Packard (Mississauga, Ont., Canada) integrator, model 3390 A. Peak identity was confirmed by comparison of retention times of standards and by gas chromatography-mass spectrometry. Mass spectra were obtained on a VG (VG Instruments Inc., Danvers, MA, USA) 70–250 mass spectrometer operated in electronic impact mode at 70 eV. The mass

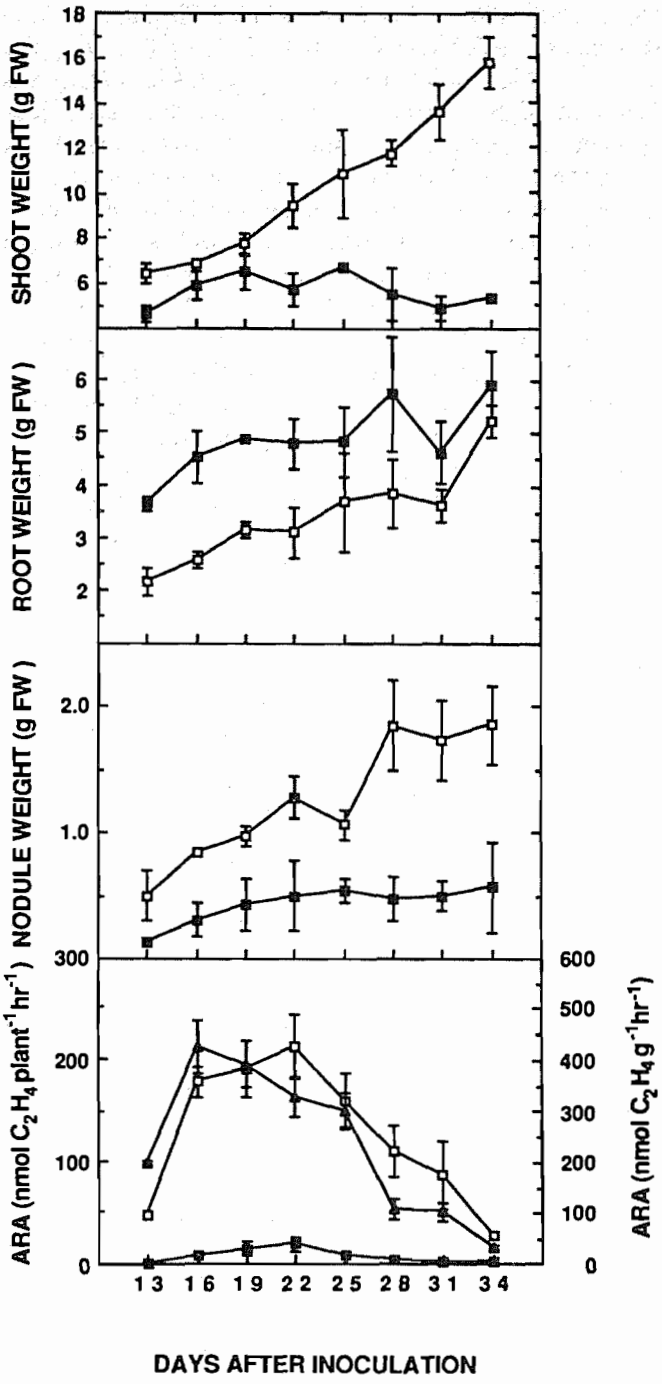

Fig. 1. Shoot, root, and nodule fresh weights for French bean inoculated with strain P_{121} (□) or strain $P_{121}S_{21}$ (■), and acetylene reduction activity by French bean inoculated with strain P_{121} (expressed on a per plant basis, □; and on a per gram fresh weight of nodule basis, ▲) or with strain $P_{121}S_{21}$ (expressed on a per plant basis, ■); measured at 3-day intervals from 13 to 34 DAI. Data points are means of 3 replicates ± SE.

spectrometer was interfaced with a Varian (Sunnyvale, CA, USA) 6000 gas chromatograph equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d.).

Data analysis

Analyses of variance were performed for all measured variables. Differences between strains for the different growth stages and strain by growth stage interactions were tested by orthogonal single degree of freedom contrasts and interaction contrasts, respectively.

Results

Plant growth

Plants inoculated with either strain P_{121} or $P_{121}S_{21}$ formed nodules and followed a similar developmental pattern, reaching flowering stage at the same time, i.e., 22 DAI. Plants inoculated with strain P_{121} established effective symbioses and were green and healthy. Plants inoculated with strain $P_{121}S_{21}$ established ineffective symbioses, were yellowish and remained smaller than those inoculated with strain P_{121}. Plants inoculated with the effective strain P_{121} increased progressively in shoot weight until 34 DAI (pod filling stage), and shoot weight was three fold greater than that of plants inoculated with strain $P_{121}S_{21}$ (Fig. 1). Plants inoculated with strain $P_{121}S_{21}$ increased in shoot weight similarly to those inoculated with strain P_{121} between 13 and 19 DAI (flowering bud formation). However, the ineffective plants did not increase in shoot weight from 19 DAI until the last sampling date, 34 DAI (Fig. 1). Root fresh weights were higher with strain $P_{121}S_{21}$ than those observed with strain P_{121}, and followed similar patterns, increasing until 34 DAI (Fig. 1).

Differences in nodulation were observed between the 2 strains. The fresh weight of nodules was higher for plants inoculated with strain P_{121} and increased until 28 DAI (pod formation) and then remained constant (Fig. 1). Nodule fresh weights for $P_{121}S_{21}$ increased between 13 and 19 DAI, and remained constant until 34 DAI (Fig. 1).

Acetylene reduction activity

ARA, whether expressed on a per plant or nodule mass basis, showed a rapid increase and reached a maximum

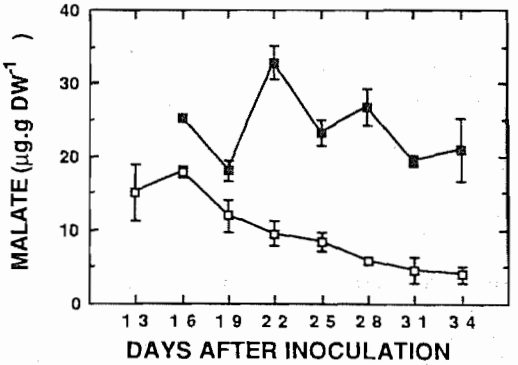

Fig. 2. Malate concentration in nodules of French bean inoculated with strain P_{121} (□) or strain $P_{121}S_{21}$ (■) measured at 3-day intervals from 13 to 34 DAI. Data points are means of 3 replicates ± SE.

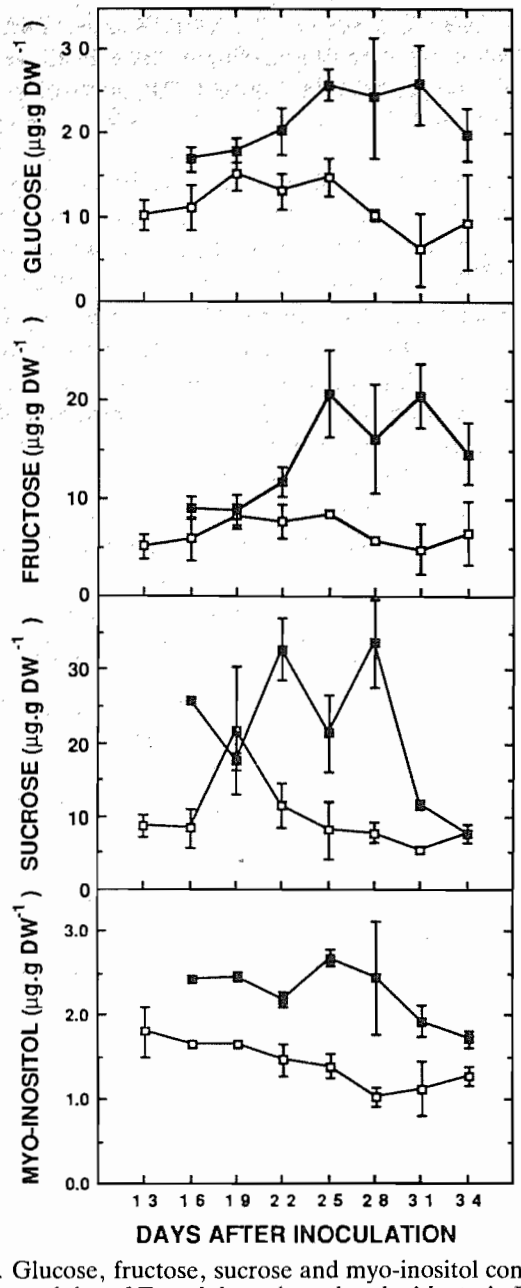

Fig. 3. Glucose, fructose, sucrose and myo-inositol concentrations in nodules of French bean inoculated with strain P_{121} (□) or strain $P_{121}S_{21}$ (■) measured at 3-day intervals from 13 to 34 DAI. Data points are means of 3 replicates ± SE.

16 to 22 DAI for plants inoculated with strain P_{121} (Fig. 1). Plants inoculated with $P_{121}S_{21}$ showed a maximum ARA less than 10% of that observed with strain P_{121} (Fig. 1).

Carbohydrate and organic acid composition of roots and nodules

The data showed that both carbohydrates and organic acids accumulated in ineffective nodules induced by strain $P_{121}S_{21}$, compared with those induced by strain P_{121} (Figs 2 to 4). The organic acid found in highest concentration in both types of nodules (P_{121} and $P_{121}S_{21}$) was malate, which varied between 3.8 and 33.2 µg (g dry weight)$^{-1}$ (Fig. 2). In effective P_{121} nodules, malate was found in highest concentration when the ARA (expressed on a per gram of nodule fresh weight basis) was the highest, and then decreased progressively until N_2 fixation had almost ceased (Figs 1 and 2). In ineffective $P_{121}S_{21}$ nodules, malate concentration followed the same pattern as in effective P_{121} nodules between 16 and 19 DAI, reached a maximum 22 DAI, and remained consistently higher than in effective P_{121} nodules until 34 DAI. Malate concentration was 10 times higher in ineffective nodules than in effective nodules 34 DAI (Fig. 2). In roots, concentrations of malate were lower than in nodules and ranged from 3.1 to 9.8 µg (g dry weight)$^{-1}$. In both P_{121} and $P_{121}S_{21}$ roots, malate concentration was highest 13 DAI and then decreased, following the same pattern until 19 DAI, and was significantly different only at 22 and 28 DAI (Fig. 2).

Malate was the only C_4-dicarboxylic acid found in high concentrations in both types of nodules. Other organic acids were detected in nodules and roots of both P_{121} and $P_{121}S_{21}$ plants. Lactate decreased in P_{121} nodules from 1.11 to 0.19 µg (g dry weight)$^{-1}$ at 13 and 28 DAI, respectively, and then was found in trace amounts until the last sampling date. In ineffective $P_{121}S_{21}$ nodules, lactate was generally higher than in effective P_{121} nodules [0.93 and 0.64 µg (g dry weight)$^{-1}$, respectively], with data averaged over all sampling dates. Lactate was always found in higher concentrations in roots than in nodules, and was also found in higher concentrations ($P < 0.05$) in roots nodulated by strain P_{121} than in roots nodulated by strain $P_{121}S_{21}$ [3.9 and 2.3 µg (g dry weight)$^{-1}$, respectively]. Usually, the concentrations of malonate, ascorbate and gluconate in effective and ineffective roots and nodules were not significantly different at different times of sampling.

Malonate also was not detected consistently and was present in very low concentrations in both types of nodules. In roots, malonate was consistently present and was generally in higher concentrations in $P_{121}S_{21}$ than in P_{121} roots [3.4 and 2.5 µg (g dry weight)$^{-1}$, respectively].

Ascorbate was detected consistently in the nodules and roots of both symbioses. Ascorbate was generally found in higher concentrations in $P_{121}S_{21}$ nodules than in P_{121} nodules [4.3 and 3.7 µg (g dry weight)$^{-1}$, respectively]. In roots, ascorbate was found in higher concentrations than in nodules, and was higher ($P < 0.05$) in $P_{121}S_{21}$ than in P_{121} roots [9.2 and 6.7 µg (g dry weight)$^{-1}$, respectively].

Gluconate was also found in high concentrations in both nodules and roots. In P_{121} nodules, gluconate was generally in higher concentrations than in $P_{121}S_{21}$ nodules [13.0 and 11.4 µg (g dry weight)$^{-1}$, respectively]. In roots, gluconate was found in lower concentrations than in nodules. Succinate and fumarate were also detected in roots and nodules, but were often found only in trace

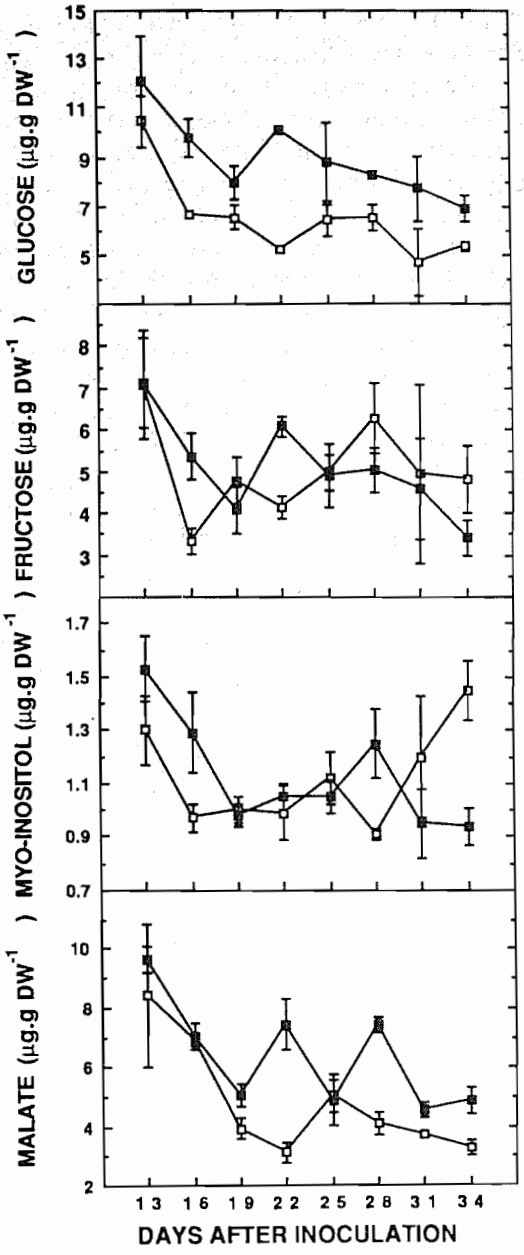

Fig. 4. Glucose, fructose, myo-inositol and malate in roots of French bean inoculated with strain P_{121} (□) or strain $P_{121}S_{21}$ (■), measured at 3-day intervals from 13 to 34 DAI. Data points are means of 3 replicates ± SE.

amounts and were difficult to quantify (data not shown).

The most prevalent carbohydrates found in both types of nodules were sucrose, glucose and fructose. In effective P_{121} nodules, sucrose reached a maximum 19 DAI, decreased until 25 DAI and then remained constant (Fig. 3). In ineffective $P_{121}S_{21}$ nodules, sucrose reached a maximum at the same time as malate (22 DAI), stayed as much as 4.5 times higher than in effective P_{121} nodules until 28 DAI (pod formation), and then decreased rapidly until 34 DAI (pod filling) (Fig. 3). Sucrose was found in trace amounts in roots and was so low that it was often difficult to quantify (data not shown). Other carbohydrates also accumulated in ineffective $P_{121}S_{21}$ nodules compared with effective P_{121} nodules. Glucose and fructose were detected in high concentrations in nodules (Fig. 3). Myo-inositol was also detected although in lower concentrations (Fig. 3). All 3 compounds reached maximum concentrations 25 DAI (full bloom). Fructose concentrations in ineffective $P_{121}S_{21}$ nodules were not significantly different from those found in effective P_{121} nodules until 22 DAI, reached a maximum 25 DAI and remained consistently higher than in effective P_{121} nodules until 34 DAI (Fig. 3). Glucose concentrations in ineffective $P_{121}S_{21}$ nodules followed the same pattern as fructose, being in significantly higher concentrations from 25 to 34 DAI (Fig. 3).

Glucose concentrations in effective P_{121} nodules remained constant until 25 DAI and decreased until 34 DAI (Fig. 3). Fructose concentrations in effective P_{121} nodules remained constant from 13 to 34 DAI (Fig. 3). Myo-inositol concentrations in ineffective $P_{121}S_{21}$ nodules remained higher than in effective P_{121} nodules until 31 DAI, and decreased to a point where myo-inositol was not significantly higher than in P_{121} nodules at 34 DAI (Fig. 3). Glucose was the only carbohydrate observed which showed significantly higher concentrations in $P_{121}S_{21}$ than in P_{121} roots for all sampling dates except at 28 and 34 DAI. From 13 to 19 DAI, glucose concentrations in P_{121} and $P_{121}S_{21}$ roots decreased rapidly and then remained rather stable until 34 DAI (Fig. 4). In both P_{121} or $P_{121}S_{21}$ roots, fructose and myo-inositol concentrations varied with no apparent clear trends (Fig. 4).

Discussion

The data reported here showed increased accumulation of carbohydrates and organic acids in ineffective nodules induced by strain $P_{121}S_{21}$ as compared with levels of these compounds in nodules induced by the effective strain P_{121}. Sucrose, the major photosynthate translocated to the nodules (Pate 1977), was found in much higher concentrations in ineffective than in effective nodules, especially between 22 and 28 DAI. The greatest differences in sucrose concentration between the 2 nodule types correspond to a period of high N_2-fixing activity for plants nodulated by strain P_{121}. The data thus indicate that even in the absence of significant N_2-fixing activity, the nodules formed by strain $P_{121}S_{21}$ nevertheless constituted a sink for incoming photosynthates. However, ineffective nodules presumably represent less active sinks than do effective nodules. Both effective and ineffective nodules require photosynthates to support growth and maintenance of nodule tissue. In addition, effective nodules also require photosynthates to support N_2 fixation and assimilation of fixed nitrogen (Pate 1977). Thus, ineffective nodules are not likely to use as much of the photosynthates for nodule respiration and nonphotosynthetic CO_2 fixation (Maxwell et al.

1984). This could explain in part the accumulation of carbohydrates and organic acids in ineffective nodules. With effective nodules formed by strain P_{121}, sucrose was probably metabolized more rapidly to support N_2 fixation, a high energy-requiring process. This could explain the low levels of sucrose found in P_{121} nodules, compared with the levels in ineffective $P_{121}S_{21}$ nodules.

$P_{121}S_{21}$ bacteroids are unable to utilize malate, fumarate or succinate as respiratory substrates (P. J. Lafontaine, C. Lafrenière and H. Antoun, unpublished results). Strain $P_{121}S_{21}$ is presumably a dicarboxylic acid transport mutant. This fact probably explained the accumulation of malate in $P_{121}S_{21}$ nodules. The accumulation of malate may have slowed the TCA cycle and glycolysis, causing the accumulation of important metabolic compounds such as sucrose, glucose and fructose. This could be an explanation for the accumulation of glucose, fructose and myo-inositol which occurred later (25 DAI) than the earlier accumulation of malate and sucrose in $P_{121}S_{21}$ nodules at 22 DAI.

There are 2 main sources of malate in nodule cells, one originating from PEP carboxylase activity and the other from glycolysis coupled to the TCA cycle via sucrose metabolism in nodules. PEP carboxylase is expressed in nodules (Jackson and Coleman 1959, Deroche et al. 1983), and one of the predominant compounds resulting from PEP carboxylase activity following $^{14}CO_2$ exposure is malate (Lawrie and Wheller 1975, DeVries et al. 1980, Coker and Schubert 1981, Gadal 1983). There is strong evidence showing that dicarboxylic acids are essential for N_2 fixation (Ronson et al. 1981, Finan et al. 1983, Arwas et al. 1985, Lafrenière et al. 1987). Among these organic acids, malate is taken up by both free-living bacteria (Finan et al. 1981, San Francisco and Jacobson 1985) and bacteroids (DeVries 1980, Reibach and Streeter 1984, Saroso et al. 1984, San Francisco and Jacobson 1985), and is one of the substrates most rapidly oxidized by bacteroids (Salminen and Streeter 1987). Bacteroids also exhibit high rates of N_2-fixing activity (C_2H_2 reduction) when provided with succinate or malate (Bergersen and Turner 1967, Trinchant and Rigaud 1981, Trinchant et al. 1981).

Malate was the predominant organic acid found in both types of nodules. Lactate and ascorbate were found in higher concentrations in $P_{121}S_{21}$ than in P_{121} nodules. The concentration of ascorbate observed in effective P_{121} nodules, which was lower than that in ineffective $P_{121}S_{21}$ nodules during periods of high N_2-fixing activity, and which showed an increase at a concentration similar to that found in $P_{121}S_{21}$ nodules 34 DAI, indicates that it may play a protective (anti-oxidant) role for nitrogenase (Dalton et al. 1986). In contrast to the situation in soybean nodules, malonate was not a major constituent of *Phaseolus* nodules (Stumpf and Burris 1981, Streeter 1987). Gluconate was also detected in both nodule types but there was no clear relationship to nitrogenase activity.

Sucrose was the predominant carbohydrate found in both types of nodules. Glucose was found in higher concentrations than fructose in both P_{121} and $P_{121}S_{21}$ nodules. These results are in accordance with those reported by Streeter (1980, 1986) and by Antoniw and Sprent (1978). The cyclitol myo-inositol was also found in nodules in lower concentrations than the other carbohydrates tested. No other cyclitols were detected in P_{121} and $P_{121}S_{21}$ nodules. These results corroborate those of Streeter (1986) and Streeter and Salminen (1985) for *Phaseolus vulgaris*. Although the role of myo-inositol and other cyclitols in nodules is not known, it has been suggested that they could enhance the activity of phytohormones (Torrey and Loomis 1967), and also participate in the formation of cell walls and cell membranes (Wolier and Murmanis 1977).

The data presented here showed that a putative C_4-dicarboxylic acid utilization mutant of *R. leguminosarum* biovar *phaseoli* is ineffective. Our data support the previous hypothesis of Ronson et al. (1981) which states that C_4-dicarboxylic acids, in this case mainly malate, are the main source of energy and reducing power for N_2-fixing bacteroids. The data also showed the accumulation of carbohydrates and organic acids in ineffective nodules formed by the mutant. The exact consequences of this accumulation on the metabolism of both the plant and the *Rhizobium* remains to be elucidated.

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